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Laminin Receptors in the Retina: Sequence Analysis of the Chick Integrin α_6 Subunit

Evidence for Transcriptional and Posttranslational Regulation

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Abstract. The integrin $\alpha_6\beta_1$ is a prominent laminin receptor used by many cell types. In the present work, we isolate clones and determine the primary sequence of the chick integrin α_6 subunit. We show that $\alpha_6\beta_1$ is a prominent integrin expressed by cells in the developing chick retina. Between embryonic days 6 and 12, both retinal ganglion cells and other retinal neurons lose selected integrin functions, including the ability to attach and extend neurites on laminin. In retinal

ganglion cells, we show that this is correlated with a dramatic decrease in α_6 mRNA and protein, suggesting that changes in gene expression account for the developmental regulation of the interactions of these neurons with laminin. In other retinal neurons the expression of α_6 mRNA and protein remains high while function is lost, suggesting that the function of the $\alpha_6\beta_1$ heterodimer in these cells is regulated by post-translational mechanisms.

DIFFERENT kinds of extracellular matrix and cell surface molecules are involved in the regulation of neuronal differentiation during the development of the nervous system. The extracellular matrix glycoprotein laminin (LN)¹ is able to stimulate neurite outgrowth by diverse peripheral and central nervous system neurons (Baron von Evercooren et al., 1982; Lander et al., 1983; Manthorpe et al., 1983; Rogers et al., 1983; Smalheiser et al., 1984; Adler et al., 1985). A developmentally regulated change in response to LN has been described for chick neural retinal cells (Cohen et al., 1986; Hall et al., 1987). Embryonic day 6 (E6) neural retinal cells attach and spread on LN, extending long neurites within 24 h in culture. In contrast, most of embryonic day 12 (E12) neural retinal cells are neither able to adhere nor to extend neurites on LN.

The mature vertebrate retina contains many different neuronal cell types. The retinal ganglion cells (RGCs) are the only cell type that extend axons out of the retina into the brain. During chick development the first RGCs extend axons towards the tectum, beginning before embryonic day 3. LN has been found to be present along the pathway followed by RGC axons, within the retina and in the optic pathway to the tectum (Mc Loon, 1984; Adler et al., 1985; Cohen et al., 1987; Halfter and Fua, 1987). The distribution of LN within the retina suggests a crucial role of this protein in axon extension during early development of the optic fiber

layer (Halfter and Fua, 1987). At later stages LN disappears from the optic pathway. At the same time, the response of RGCs to LN when assayed in vitro decreases. By E12 most of the RGC axons have reached the tectum (Rager, 1980), and this seems to correlate with the loss of the ability of many E12 retinal cells to spread and grow neurites on LN in vitro (Hall et al., 1987).

The involvement of an integrin receptor in the adhesion of neural retinal cells to LN is indicated by the fact that the monoclonal antibody CSAT against the chick integrin β_1 subunit is able to inhibit virtually all cell attachment and neurite outgrowth by E6 neural retinal cells and RGCs on LN in vitro (Buck et al., 1986; Cohen et al., 1987; Hall et al., 1987). The same cells continue to express integrins and remain able to bind at least some extracellular matrix constituents (Hall et al., 1987). Since these neurons exhibit changes during development in their ability to interact with LN, integrin function in these cells must be regulated.

Integrins are noncovalently associated $\alpha\beta$ heterodimers which represent the primary receptors used by many cells to interact with the extracellular matrix (see Hemler, 1990, for a review). At least seven different α subunits associate with the β_1 subunit to form receptors with different ligand binding specificities. Four of these α subunits, α_1 , α_2 , α_3 , and α_6 , form $\alpha\beta$ heterodimers able to function as LN receptors.

The aim of the present study was to examine the expression and the regulation of LN-binding integrins in the developing chick retina. Using a panel of antibodies to α_1 , α_2 , α_3 , and α_6 , we found that α_3 , and α_6 are expressed by de-

1. Abbreviations used in this paper: CMF-PBS, Ca^{++} - and Mg^{++} -free PBS; LN, laminin; RGC, retinal ganglion cell.

veloping retinal cells. α_6 levels were seen to decrease with age, suggesting that downregulation of α_6 expression accounts in part for the loss of responsiveness to LN. We isolated and sequenced clones encoding the chick α_6 subunit by using a cDNA clone of the human α_6 subunit (Tamura et al., 1990). Using the α_6 cDNA, and specific antibodies, we have examined the developmental expression of this protein at the transcriptional and translational level. Upon fractionation of retinal cells on a density gradient, we have found that α_6 expression is most dramatically regulated in the highly purified fraction of RGCs.

Materials and Methods

Reagents and Solutions

White leghorn chicken eggs were purchased from Feather Hill Farm (Petaluma, CA). Nitrocellulose filters were from Schleicher & Schuell Inc. (Keene, NH). Restriction enzymes and Klenow fragment of DNA polymerase were from New England Biolabs (Beverly, MA) and Boehringer Mannheim Diagnostics, Inc. (Houston, TX). Sequence enzyme, reagents for sequencing, and random hexanucleotide primers were from kits supplied by U.S. Biochemical Corp. (Cleveland, OH). [α - 35 S]dATP and [γ - 32 P]dCTP were from Amersham Corp. (Arlington Heights, IL). D-[6- 3 H(N)]-glucosamine hydrochloride and EN 3 HANCE were from NEN, DuPont Co. (Wilmington, DE). Percoll, protein A-Sepharose CL-4B, and CNBr-Sepharose CL-4B were from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Protein determination was performed according to Bradford (1976) using a Bio-Rad kit (Bio-Rad Laboratories, Richmond, CA). Denhardt's solution (1 \times) is 0.02% Ficol type 400, 0.02% BSA, 0.02% polyvinylpyrrolidone.

Isolation of cDNA Clones

An embryonic day-10 chicken cDNA library in λ gt10 was obtained from Dr. C. Nottenburg (Fred Hutchinson Cancer Research Center, Seattle, WA), and an embryonic day-13 chicken brain library in λ gt10 was obtained from Dr. B. Ranscht (Cancer Research Institute, La Jolla, CA). The libraries were plated on *Escherichia coli* strain Y1089, and replica filters were screened at low stringency according to a modified procedure of Church and Gilbert (1984). Prehybridization were carried out for 7 h at 38°C in 7% SDS, 15% formamide, 1 mM EDTA, 1% BSA, 0.01% salmon sperm DNA, 0.2 M sodium phosphate buffer, pH 7.0. Hybridizations were performed for 12 h at 38°C in fresh aliquots of the same buffer with the denatured probes. The probes were prepared from gel-purified restriction fragments using the random priming method of Feinberg and Vogelstein (1983), using 50 μ Ci of [γ - 32 P]dCTP for each probe. The phage library screenings involving high stringency hybridization were performed as described in Maniatis et al. (1982). The first screening of the chicken cDNA libraries performed at low stringency were performed using three gel-purified restriction fragments from a human α_6 cDNA clone (Tamura et al., 1990). The filters were washed five times for 15 min at 45°C in 0.1% SDS, 300 mM sodium chloride, 30 mM sodium citrate, pH 7.0, and one time for 2 min in 30 mM sodium chloride, 3 mM sodium citrate, pH 7.0, before autoradiography.

DNA Sequencing

cDNAs were subcloned in M13 mp18 or mp19 (Messing, 1983). Sequencing was according to the dideoxy chain termination method (Sanger et al., 1977) from their extremities, and by extension using specific oligonucleotides. The nucleotide sequence displayed in Fig. 5 has been determined on both strands of two independent and overlapping clones, by reconstituting a linear sequence from overlapping stretches. The nucleotide and amino acid sequence analyses were performed with the PCGENE programs (Intelligenetics Corp., Mountain View, CA).

Antibodies and Protein Purification

Hybridoma cells secreting CSAT antibodies were a kind gift from Dr. A. F. Horwitz, University of Pennsylvania, Philadelphia, PA, and were grown as described (Neff et al., 1982). Antibodies were purified from ascites on protein A-Sepharose CL-4B columns as previously described (Hall et al., 1987). The monoclonal antibody against chick G4 protein was a kind gift

of Dr. F. Rathjen, Zentrum für Molekulare Neurobiologie, Hamburg, Federal Republic of Germany.

Oligopeptides corresponding to the last 35 carboxy-terminal amino acids of the human α_6 protein, to an amino-terminal cysteine plus the last 24 carboxy-terminal amino acids of the chicken α_3 integrin (Hynes et al., 1989), and to an amino-terminal cysteine plus the last 15 carboxy-terminal amino acids of the rat α_1 integrin (Ignatius et al., 1990) were synthesized at the Howard Hughes Medical Institute by Dr. C. Turk. 34 of the 35 residues of the human α_6 peptide are identical to the chick α_6 cytoplasmic tail. The peptides were coupled to sulfo-MBS (Pierce Chemical Co., Rockford, IL) activated KLH (Calbiochem-Behring Corp. San Diego, CA). Rabbits were immunized with the KLH-peptide complex in Freund's complete adjuvant and boosted with the antigen in Freund's incomplete adjuvant at Caltag Laboratories (South San Francisco, CA). The α_1 - and α_6 -specific antibodies were affinity purified on a peptide-thiopropyl-Sepharose CL-4B column, while an IgG fraction was prepared from the anti- α_3 serum. An affinity-purified polyclonal antibody against the last 12 carboxy-terminal amino acids of the human α_2 integrin was kindly provided by Dr. John A. McDonald (Washington University Medical Center, St. Louis, MO). The polyclonal antibodies against the chicken integrin β_1 subunit were raised against the last 23 carboxy-terminal residues of this protein as described by Tomaselli et al. (1988).

LN was purified from Engelbreth-Holm-Swarm sarcoma as published (Timpl et al., 1979).

RNA Analysis

Total RNA from E6 and E12 retinæ or from cellular fractions thereof (prepared as described in the next section) was prepared according to Chomczynski and Sacchi (1987). 7.5–20 μ g of total RNA for each sample were electrophoresed using standard methods as described by Maniatis et al. (1982) and transferred overnight by capillary action to nitrocellulose filters. RNA blots were probed using standard hybridization techniques (Bossy and Reichardt, 1990) with 50 ng of the gel purified 2.5 kb α_6 cDNA fragment C5 (see Fig. 4) which had been labeled according to Feinberg and Vogelstein (1983). RNA blots with same amounts of total RNA were probed in parallel with a random labeled actin probe. Quantitation of bands from autoradiograms was made according to Suissa (1983). The values for the α_6 transcript were normalized to the corresponding values for the actin transcript, used as a control.

Retinal Ganglion Cells Isolation and Neurite Outgrowth Assay

After dissection, E6 and E12 retinæ were incubated for 5 min at 37°C in 0.2% hyaluronidase in Ca $^{++}$ - and Mg $^{++}$ -free PBS (CMF-PBS; PBS is 0.2 g/liter KCl, 0.2 g/liter KH $_2$ PO $_4$, 2.16 g/liter Na $_2$ HPO $_4$, 8 g/liter NaCl, 0.1 mM CaCl $_2$, 0.1 mM MgCl $_2$) tissue pellets were resuspended in 0.1% trypsin (Worthington Biochemical Corp., Freehold, NJ), 0.002% DNase I in CMF-PBS, and digested for 8 min at 37°C. Digestion was stopped by adding 0.2 \times vol fetal calf serum. The pellets were washed once in F12 nutrient mixture, and triturated in F12 containing 0.2% soybean trypsin inhibitor, 0.002% DNase I.

RGCs were isolated according to a modified procedure kindly provided by Dr. J. E. Johnson, University of Arkansas, Little Rock, AR (personal communication). The cell suspension obtained from the trypsinized retinæ was loaded on a discontinuous Percoll gradient prepared in a 50 ml tube as follows: the bottom layer ($d = 1.090$) was obtained by mixing 4 ml of F12 medium and 5.65 ml of Percoll; the second layer ($d = 1.060$) was obtained by mixing 6 ml of cell suspension in F12 with 3.8 ml of Percoll; the third layer ($d = 1.038$) was obtained by mixing 8 ml of cell suspension in F12 medium with 1.75 ml Percoll; the fourth layer ($d = 1.017$) was obtained by mixing 8 ml of cell suspension in F12 medium with 0.84 ml Percoll; the top layer was 9 ml of F12 medium. The gradients were centrifuged at 4°C for 30 min at 1,800 rpm in a Sorvall HB-4 rotor (DuPont, Wilmington, DE). The two visible bands were collected separately and washed once in F12 medium, and resuspended in F12 medium with additives (5 μ M/ml insulin, 30 nM selenium, 25 μ g/ml iron-saturated ovotransferrin, 100 μ g/ml human transferrin, 100 U/ml penicillin and streptomycin, according to Bottstein et al., 1980). We called fraction I the lower density band ($d = 1.017/1.038$) and fraction II the higher density band ($d = 1.038/1.060$). Fraction I contained about 5% of the protein contained in the fraction II. Fraction I corresponded to a cell population enriched in RGCs. Typically 30–40 \times E6 retinæ or 10–15 \times E12 retinæ were used for each preparation.

For the neurite outgrowth assay retinal cells or cells from fraction I and

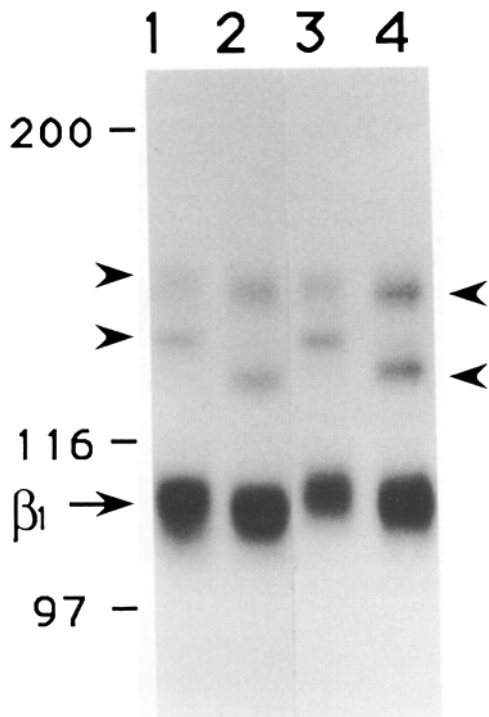


Figure 1. Immunoprecipitation of retinal cells with anti- β_1 antibodies. Retinal cells were prepared by trypsinization of E6 (lanes 1 and 3) and E12 (lanes 2 and 4) chick retinae, as described in Materials and Methods. Cells were metabolically labeled for 20 h in the presence of [3 H]glucosamine. For immunoprecipitation the same amount of TCA-precipitable 3 H counts were used for each lane. Immunoprecipitates were separated on 6% SDS-PAGE under nonreducing conditions, and the gel processed for fluorography. Lanes 1 and 2, immunoprecipitates with antibody against the cytoplasmic tail of β_1 ; lanes 3 and 4, immunoprecipitates with the β_1 -specific monoclonal antibody CSAT. The arrowheads indicate the major polypeptides coprecipitated together with β_1 .

II of the Percoll gradient were plated at low density on glass coverslips previously coated with 20 μ g/ml LN, as previously described (Hall et al., 1987). After 12-h incubation at 37°C in a 5% CO₂ atmosphere, the cultures were fixed with 3% paraformaldehyde in PBS and examined in the microscope.

Cell Attachment Assay with Retinal Cells

E6 and E12 retinae were dissected and dissociated as described in the previous section. Linbro/Titer plates (Flow Laboratories, Inc., McLean, VA) were coated overnight with 25 μ g/ml of LN, or with 1% poly-D-lysine in PBS used as a positive control. Coated and uncoated (negative control) wells were incubated for 2 h at room temperature with 1% BSA in PBS. Wells were washed twice with PBS and ~200,000 retinal cells were added to each well. Cells were incubated 1.5 h at 37°C in 5% CO₂ atmosphere. Unattached cells were removed by the brisk addition of warm F12 medium followed by gentle vacuum suction. The cells were fixed 10 min with 2% glutaraldehyde. Cells were stained with crystal violet (0.5% in 20% methanol) and washed with water (Bodary et al., 1989). After solubilization with 1% SDS, A₅₄₀ was measured in each well. The percentage of cells attached to LN-coated wells was calculated as described by Hall et al. (1987).

Metabolic Labeling and Immunoprecipitation

Dissociated retinal cells were metabolically labeled by incubation for 20 h at 37°C in 5% atmosphere in F12 medium with additives containing 250 μ Ci/ml of [3 H]glucosamine. After labeling, the cells were removed from the dish by gentle pipetting. When adherent cells were present, they were harvested using a rubber policeman. The cells were washed in PBS and the

pellets were resuspended in 5–10 \times vol lysis buffer (1% Triton X-100 in PBS, containing 5 μ g/ml of antipain, chymostatin, leupeptin, and pepstatin). Lysates were spun for 10 min at 12,000 rpm in a refrigerated microfuge, and the pellets were discarded. [3 H]Glucosamine incorporation into protein was determined after precipitation of aliquots of the lysates with 10% trichloroacetic acid, by counting on an LS 3801 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Aliquots of the lysates were incubated overnight at 4°C with 10 μ l of antiserum or 10–40 μ g of affinity-purified antibodies. Antigen-antibody complexes were precipitated with protein A-Sepharose CL-4B beads for 1.5 h at 4°C. Immunoprecipitates were washed eight times with lysis buffer and processed for SDS-PAGE as described below. Fixed gels were treated with EN³HANCE, dried, and exposed to Kodak XAR-5 film.

SDS-PAGE and Immunoblotting

Retinal cells were incubated overnight at 37°C in 5% CO₂ atmosphere in F12 medium with additives to recover from trypsinization. Cells were harvested and lysates were prepared as described in the previous section. Where indicated, whole retina samples were used. In this case, the dissected retinae were immediately solubilized in lysis buffer. The lysates were mixed with electrophoresis sample buffer and boiled for SDS-PAGE analysis (Laemmli, 1970). Samples were run on 6% acrylamide gels under reducing or nonreducing conditions.

For Western blot analysis proteins were electrophoretically transferred from gels to nitrocellulose filters for 1–2 h at 500 mA. The filters were blocked in blocking buffer (5% nonfat dry milk in 100 mM Tris-Cl, pH 7.5, 150 mM NaCl). Primary antibodies were diluted to 5–20 μ g IgG/ml in blocking buffer and the filters were incubated for 1 h at room temperature (anti- α integrins polyclonal antibodies) or overnight at 4°C (anti-G4 monoclonal antibody). The filters were washed four times for 10 min in blocking buffer. 0.1 μ Ci/ml of 125 I-labeled anti-rabbit IgG antibodies (ICN Biomedicals Inc., Irvine, CA) or 0.5 μ g/ml alkaline phosphatase-conjugated anti-mouse IgG antibodies (Promega Biotec, Madison, WI) were added, and the filters were incubated for 1 h at room temperature. Filters incubated with 125 I-labeled antibodies were washed three times for 10 min with blocking buffer containing 0.1% Triton X-100 and 0.02% SDS and once with CMF-PBS. The filters were dried and exposed to Kodak XAR-5 films. Quantitation of bands from autoradiograms was made according to Suissa (1983). Filters incubated with alkaline phosphatase-conjugated antibodies were washed four times for 10 min in blocking buffer and developed with 0.33 mg/ml nitro blue tetrazolium and 0.16 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in 0.1 M Tris-Cl, pH 9.0, 0.1 M NaCl, 5 mM MgCl₂.

Results

Differential Expression of Integrin α Subunits during Retinal Development

To identify the putative α subunits that might be involved in the changes in affinity of retinal cells for LN during development, we used the monoclonal antibody CSAT against an extracellular epitope of the integrin β_1 subunit and a polyclonal antibody against the cytoplasmic domain of the chicken integrin β_1 subunit to immunoprecipitate cell extracts from E6 and E12 retinal cells metabolically labeled with [3 H]glucosamine after dissociation with trypsin. Fig. 1 shows that the pattern of α subunits immunoprecipitated with both antibodies was the same in retinal cells from the same developmental stage. Comparison of the pattern of α subunits associated with the β_1 polypeptide showed clear differences between the two developmental stages. Two major bands of M_r 150 and 140 kD were coprecipitated with β_1 from E6 cell extracts (Fig. 1, lanes 1 and 3); immunoprecipitates from E12 cell extracts showed two major bands at M_r 150 and 130 kD, while the intensity of the 140-kD band was strongly reduced (Fig. 1, lanes 2 and 4). Both E6 retinal neurons and RGCs have been shown to grow long neurites when cultured on LN, while E12 retinal cells of both types have been shown

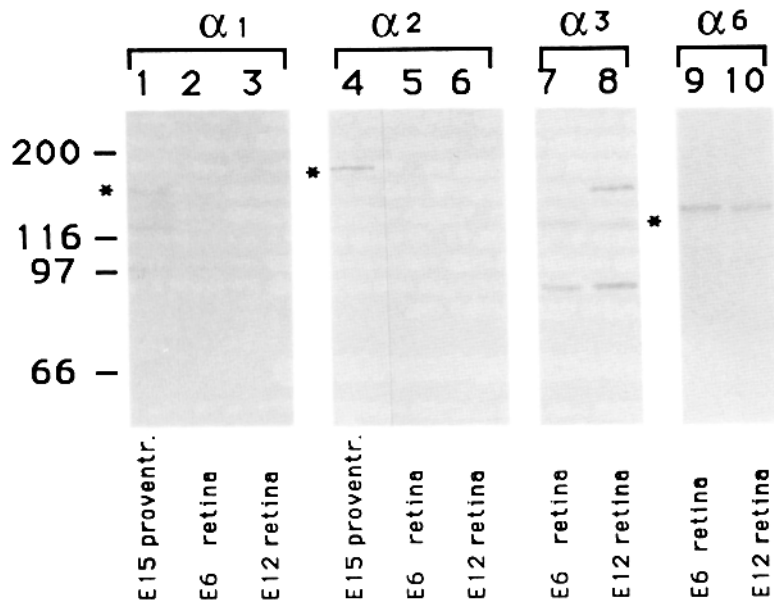


Figure 2. Immunoblot analysis for the presence of different α integrin in retina. E15 proventriculum (lanes 1 and 4), E6 retinae (lanes 2, 5, 7, and 9), and E12 retinae (lanes 3, 6, 8, and 10) were dissected and extracted as described in Materials and Methods. 120 μ g protein from the extracts was loaded in each lane and electrophoresed. Blots were incubated with polyclonal antibodies against the integrins α_1 (lanes 1–3), α_2 (lanes 4–6), α_3 (lanes 7 and 8), and α_6 (lanes 9 and 10), followed by incubation with an alkaline phosphatase-conjugated anti-rabbit IgG antibody. Asterisks indicate the bands corresponding to the different α subunits. The lower and higher molecular weight bands observed in lanes 8 and 9 were also present in the preimmune serum and could not be competed by the α_3 -derived peptide used for the immunizations, when the blots were incubated in the presence of this peptide during the incubation with the anti- α_3 antibody (not shown).

to extend no neurites on LN (Cohen et al., 1986; Hall et al., 1987). Neurite outgrowth of E6 cells on LN has been shown to be blocked completely by the monoclonal antibody CSAT.

Among the known integrin α subunits, α_1 , α_2 , α_3 , and α_6 in association with the β_1 subunit can all function as LN receptors in different cell types (Hemler, 1990). We analyzed E6 and E12 retinal extracts by immunoblot using poly-

clonal antibodies specific for these different α subunits. The α_1 and α_2 integrins were not detectable in E6 and E12 retinae, while they were present in E15 chick proventriculus (Fig. 2, lanes 1–6). Both α_3 and α_6 subunits were present in retinae at both developmental stages (Fig. 2, lanes 7–10), but only the α_6 subunit appeared to be down regulated in E12 retinae compared with E6 retinae. When metabolically labeled retinal cell extracts were immunoprecipitated with a polyclonal antibody against a peptide corresponding to the 35 carboxy-terminal amino acids of the cytoplasmic tail of the integrin α_6 subunit, two polypeptides were visible on the gel (Fig. 3, lanes 2 and 3): a lower relative molecular mass protein which comigrated with the β_1 subunit, and a polypeptide of 140 kD corresponding to the α_6 subunit. The α_6 polypeptide comigrated with the α subunit that ap-

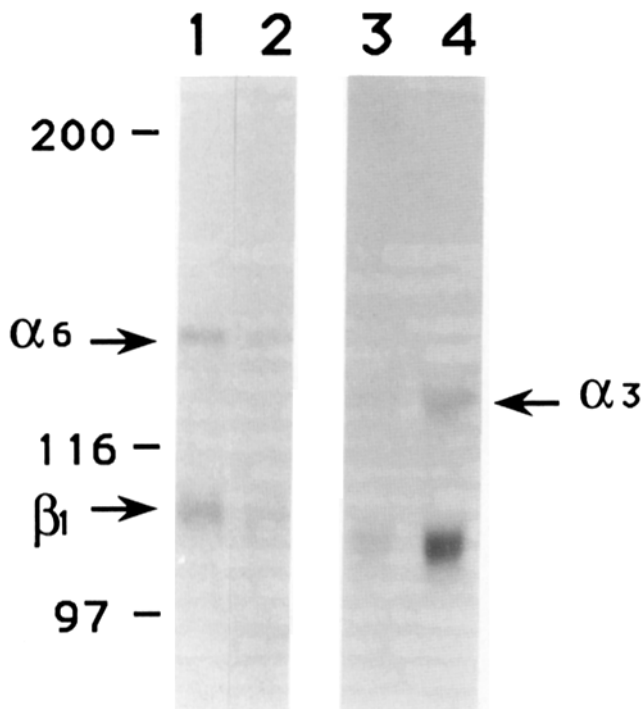


Figure 3. Immunoprecipitation of retinal cells with anti- α_6 and anti- α_3 antibodies. Immunoprecipitations with anti- α_6 (lanes 1 and 2) and anti- α_3 (lanes 3 and 4) antibodies from metabolically labeled E6 (lanes 1 and 3) or E12 (lanes 2 and 4) retinal cells were performed as described in Fig. 1. The same amount of TCA-precipitable 3 H counts was used for each immunoprecipitation in lanes 1 and 2, and in lanes 3 and 4.

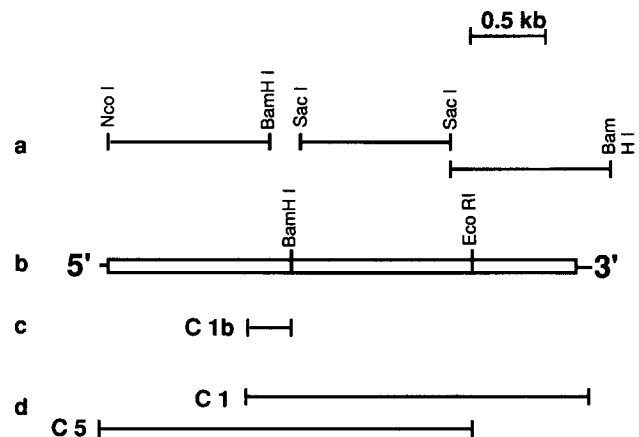


Figure 4. Scheme of the probes and purified cDNA clones. (a) Restriction fragments purified from human α_6 cDNAs used as probes for screening at low stringency. (b) Restriction map of the chick α_6 sequence obtained by combining the two overlapping cDNAs C1 and C5 (d), obtained after screening of the E10 chick λ gt10 library and of the E13 chick brain λ gt10 library, respectively. The open box represents the main open reading frame. (c) EcoRI-BamHI restriction fragment from the C1 chick α_6 cDNA used as probe to screen at high stringency the E13 chick brain λ gt10 library.

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CTCGGGCGCAGGTTCTCGTCCGCGCTGGAGCAGATGGCGGCGCGCTCTCTCTACCTGCCCCCTGCTCCGGGGCTGGCGGCGCTTCAACCTGGAC 99
      M A A A L L L Y L P L L P G L A G A F N L D 4

CGGAGAACGTGATCGCGCGCGCGGAGCGCGGCGCCTCTTCGGCTTCTCGCTGGCCATGCACCGCAGCTGCAGCCGAGGAGAAACGGCTGTGCTGGTT 204
A E N V I G R R G E P G S L F G F S L A M H R Q L Q P Q E K R L L L V 39

GGAGCTCTCTCGGAGAAAGCGCTTCCATCCCAACGCAACAGAGCGGAGGGCTGTACAGCTGTGACATCAATCTCAGATACGGCTGCACGGCTGTGTG 309
G A P R E K A F P S Q Q A N* R T G G L Y S C D I T S S D T R C T R V V 74

TTTGATGAAGACTGACCCAAAGATGGAGAGCAAGAACCAATGGATGGGTGAATGTCCAAAGTCAGGGCCAGGAGAAACGGTGGTACATGTGCACAT 414
F D E D T D P K M E S K E D Q W M G V T V Q S Q G P G G N V V T C A H 109

CGCTATGAGAAAGCAATATGTAACACAGTCGAGGAAACCGGATATCATTTGGAAGTGTCTGCTGAGCCAGGATCTCACTATTAAGGATGATATGGAT 519
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M T F V S G A P R A N* H S G A V V L L K K E K N Q R A L S L E H M F E 284

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K V N V D T T D L D I N L K L E T T S T Q V N* S T A I T A S A K V V L 774

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E L L L S L T G V A K P S Q V Y F G G N I V G E S A M K S E D N I G N 809

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L I E Y E F R V T N L G R P L K T F G T A S L D I Q W P K E I S N G K 844

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M L L Y L M K I E S K G L E K V S C Q P Q N E I N V L H V A E S H N S 879

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R R K R E I A E K Q L T D S K T F S L F S E R K Y K T L D C K V N A Q 914

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C G D I R C I F L K G F D S K A I L R S R L W N* S T F L E E F S K M 949

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N Y L D I L V R A S I S V P A A A K N V K L T N E A A Q V R V T V F P 984

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A K P V A L Y T G V P W W I I A V A I F A G V L M L A L L V F L L W K 1019

TGTGTTTCTTCAAAAGAGTAAGAAAGATCATACGATGCCACATATCACAAGGCTGAGATCCATGCTCAGCCATCTGATAAAGAGAGGCTTACTTCTGATGCG 3249
C G F F K R S K K D H Y D A T Y H K A E I H A Q P S D K E R L T S D A 1054

TAGTATTGATCTACTCTGTAATTTGTGGGTTCTTCCAGGTTCCAGGTACGATGACAGCGTCCCGGCTATCATGCTGTAA 3332

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Figure 5. Complete nucleotide sequence of chick α_6 as deduced from the C1 and C5 cDNA clones and its translated amino acid sequence. The arrow points to the start of the mature polypeptide, which is preceded by 18-residue-long signal peptide. Probable calcium binding sites are indicated by boxed areas and the transmembrane domain by a solid line. Asterisks mark the possible N-glycosylation sites. These sequence data are available from EMBL/GenBank/DBJ under accession number X56559.

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1
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LQVKSCFEYTANPAGYNPISIVGTLEAEKERRKSGLSRVQFRNQSEPKYTQELTLKRQKQVCMETLWLQDNIRDKLRPIPIAVSVNIAGLESQSS 569
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Figure 6. Amino acid sequence comparison between chicken and human α_6 . The amino acid sequences for chicken (top line) and human (bottom line) α_6 have been aligned, and the residues conserved between the two species are marked by a vertical bar between the two sequences. The predicted cleavage sites for the signal sequence (between residues -1 and 1) and within the extracellular domain (between residues 883 and 884) are indicated by vertical arrows, the membrane spanning regions are underlined and the putative calcium binding domains are enclosed in boxed areas. The consensus phosphorylation site for Ca^{++} -calmodulin-dependent protein kinase II is indicated by the dots on amino acid 1,048-1,051 of the chick sequence.

peared to be down regulated in the β_1 immunoprecipitates from retinal cells between E6 and E12. When the extracts from labeled retinal cells were immunoprecipitated with the anti- α_3 antibody, no down regulation of this integrin was observed between the two developmental stages (Fig. 3, lanes 3 and 4).

Cloning of the Chick α_6 Subunit and Sequence Analysis

The immunocytochemical data suggest that α_6 could be the α subunit responsible for the developmentally regulated ability of retinal cells to adhere and grow neurites on LN. To be able to study the expression of this α subunit during retinal development, we isolated clones encoding the chick α_6 subunit. We used three restriction fragments (Fig. 4) containing most of the translated sequence of the human integrin α_6 subunit (Tamura et al., 1990) to screen an embryonic day-10 chick λ gt10 library at low stringency (T_m -50°C). Three overlapping clones were purified and sequenced. None of these clones contained the 5' end of the translated portion of the gene. To obtain the missing sequence, we screened an embryonic day-13 chick brain λ gt10 library. The library was screened at low stringency using the three restriction fragments from the human cDNA, and at high stringency using an EcoRI/BamHI restriction fragment (C1b) corresponding to the most 5' portion of the chick cDNA clone C1. Three more cDNA clones were isolated, purified and sequenced. Clone C5 contained the 5' end portion of the gene together with 33 bp of the 5' nontranslated region.

Fig. 5 shows the nucleotide and amino acid sequence of

the chick protein predicted by translation of the longest open reading frame. The cDNA sequence includes 3,216 nucleotides of open reading frame and 33 and 83 nucleotides of the 5' and 3' untranslated regions, respectively. The deduced amino acid sequence corresponds to a protein with 1,072 amino acid residues, with a 73% identity to the sequence of the recently published human α_6 subunit (Tamura et al., 1990). This high amino acid sequence conservation is much higher than the homologies observed between the chick α_6 integrin and several other chicken or mammalian α subunits (<40% identity). The homology to the human α_6 subunit becomes higher if we consider the NH_2 -terminal portion of the extracellular domain, and the cytoplasmic tail, where 35 over 36 amino acid residues are identical. Therefore this sequence seems to correspond to the chick homologue of the human α_6 integrin subunit. A putative cleavable signal peptide of 17 amino acids after the starting methionine is present at the NH_2 terminus of the polypeptide. The protein contains a large extracellular domain, a single transmembrane domain and a short cytoplasmic tail (36 amino acids) that represents the region of highest homology with the human α_6 polypeptide (Fig. 6). This includes a consensus phosphorylation site RXXS (amino acid 1,049-1,052) for Ca^{++} -calmodulin-dependent protein kinase II (Kemp and Pearson, 1990), which is of interest, since α_6 can be phosphorylated in vivo (Shaw et al., 1990). The presence of 10 potential N-glycosylation sites (N-X/non P-T/S) predicted to contribute a total of 25,000 D to the relative molecular mass of the core protein (Parham et al., 1977) would yield a predicted relative molecular mass for the mature protein of 142 kD on SDS-

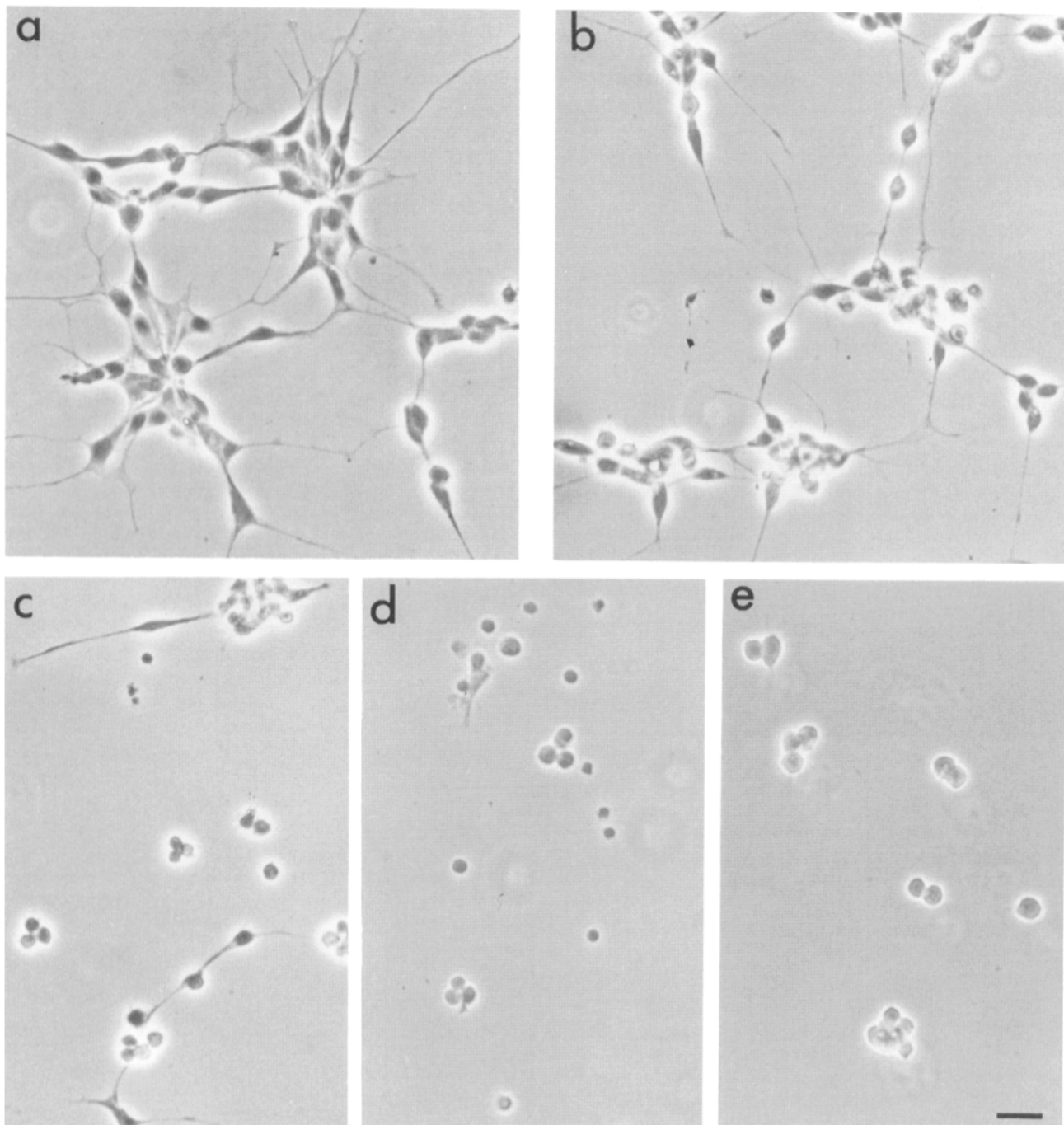


Figure 7. Phase-contrast microscopy of cellular fractions I and II from Percoll gradients. After trypsinization, E6 (*a*, *b*, and *e*) and E12 (*c* and *d*) retinal cells were fractionated on Percoll gradients as described in Materials and Methods. Cells from fractions I (*b* and *d*) and II (*a* and *c*) were incubated overnight on LN-coated glass coverslips in defined medium. In *e* E6 cells from fraction I were cultured on LN in the presence of 10 µg/ml of the monoclonal antibody CSAT. Bar, 50 µm.

PAGE, which is in agreement with the relative molecular mass of the polypeptide recognized under nonreducing conditions on immunoblots or found on SDS-PAGE after immunoprecipitation by a polyclonal antibody raised against the cytoplasmic domain of the α_6 protein (see Fig. 3). 17 of the 19 cysteines present in the extracellular domain of the mature human α_6 polypeptide are conserved in the chick subunit. Three putative calcium binding sites are present in

the extracellular domain, and a putative cleavage site between residues 883 and 884 which satisfies the consensus (K/R-R-E/D) observed so far among other integrin α subunits (Hemler, 1990). On immunoblots from retinal cell lysates fractionated on SDS-PAGE under reducing conditions only a low relative molecular mass polypeptide of ~23 kD was specifically recognized by the anti- α_6 antibody, suggesting that the protein is cleaved (data not shown).

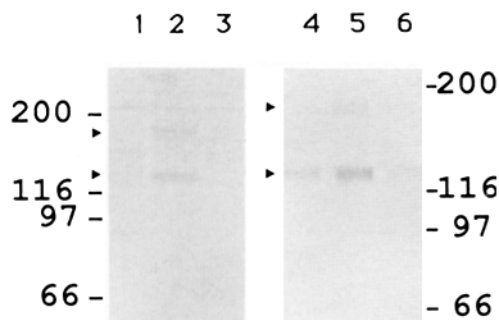


Figure 8. Distribution of G4 antigen between fractions I and II of Percoll gradients. E6 (lanes 1–3) and E12 (lanes 4–6) retinal cells were trypsinized, fractionated on Percoll gradients, and cultured overnight in defined medium to recover from trypsinization. After detergent extraction of the different cellular fractions, aliquots corresponding to 100 μ g of protein from total retinal cells (lanes 1 and 4), and from fractions I (lanes 2 and 5) and II (lanes 3 and 6) of the Percoll gradients were separated on 6% SDS-PAGE under reducing conditions and blotted onto nitrocellulose. Blots were incubated with the monoclonal antibody G4, followed by an alkaline phosphatase-conjugated anti-mouse IgG antibody. Arrowheads point to the major bands specifically recognized by the G4 antibody.

Isolation of Retinal Ganglion Cells

The RGCs are the only retinal neurons extending their axons out of the retina, along the optic nerve, where LN has been shown to be transiently expressed during development. To characterize changes in α_6 subunit expression on RGCs specifically, we wanted to isolate a fraction enriched in RGCs. A cellular population enriched in RGCs was obtained by fractionation of E6 or E12 retinal cells on a discontinuous Percoll gradient, as described in Materials and Methods. Two cellular fractions were separated from the gradients after centrifugation: fraction I corresponded to a broad and fainter band which was found on the upper part of the gradient ($d = 1.017$ – 1.038), and fraction II consisted of the higher density band ($d = 1.038$ – 1.060) where most of the cells were recovered after centrifugation. The two fractions had similar densities when either E6 or E12 retinal cells were used. When cells from fractions I and II from Percoll gradients loaded with E6 retinal cells were cultured overnight on LN-coated coverslips, most of the cells extended neurites (Fig. 7, *a* and *b*). Nevertheless, the overall morphology of the two cultures was significantly different. Several cells from the fraction II of the gradient showed a polygonal shape, while most of the cells from the fraction I of the gradient had a round cell body and long neurites, which were eliminated by the presence of 10 μ g/ml of the CSAT antibody (Fig. 7 *e*). This different morphology suggested that two different retinal cell populations had been separated on the Percoll gradient. When cells from the two fractions from a gradient loaded with E12 retinal cells were cultured overnight on LN, only very few cells from both fractions I and II attached on LN, and these cells did not extend virtually any neurites (Fig. 7, *c* and *d*).

To follow the distribution of RGCs in the two gradient fractions, we used the monoclonal antibody G4 (Rathjen et al., 1987). This monoclonal antibody recognizes a set of polypeptides which are related to the mouse L1 surface antigen (Rathjen et al., 1987). This protein has been shown to be mostly concentrated on the surface of RGCs in the devel-

oping chick retina (Lemmon and McLoon, 1986). The immunoblot in Fig. 8 shows that the G4 antibody recognized a polypeptide of 140 kD and a doublet of ~ 190 kD in retinal cell preparations. The G4 antigen was clearly enriched in fraction I from both E6 and E12 retinal cells (Fig. 8, lanes 2 and 5) compared with the total retinal cell population (Fig. 8, lanes 1 and 4) and to the cells recovered from fraction II (Fig. 8, lanes 3 and 6), indicating an enrichment in RGCs in fraction I. From this point fraction I from the Percoll gradients will be referred to as the RGC fraction. Fraction II represents the RGC-depleted fraction.

Developmental Regulation of Integrin α_6 Subunit in RGC

To determine whether the integrin α_6 subunit expression is developmentally regulated in RGCs, RNA blots of total RNA prepared from total retinae and from the cellular fractions from the Percoll gradients were probed with the random primed 32 P-labeled cDNA clone C5 of the chicken α_6 . A single band corresponding to a 5.3-kb transcript was detected (Fig. 9, *top*). A strong reduction in the amount of α_6 mRNA was observed in RGC fraction from E12 retinae compared to the RGC fraction prepared from E6 retinae (Fig. 9, lanes 3 and 4). Quantitation of the bands from autoradiograms was made by normalizing the values obtained from α_6 to the corresponding values for actin, used as a standard. The quantitation showed that the amount of α_6 mRNA in the E12 RGC fraction was 25% of that present in the E6 RGC fraction. We think that the percentages of α_6

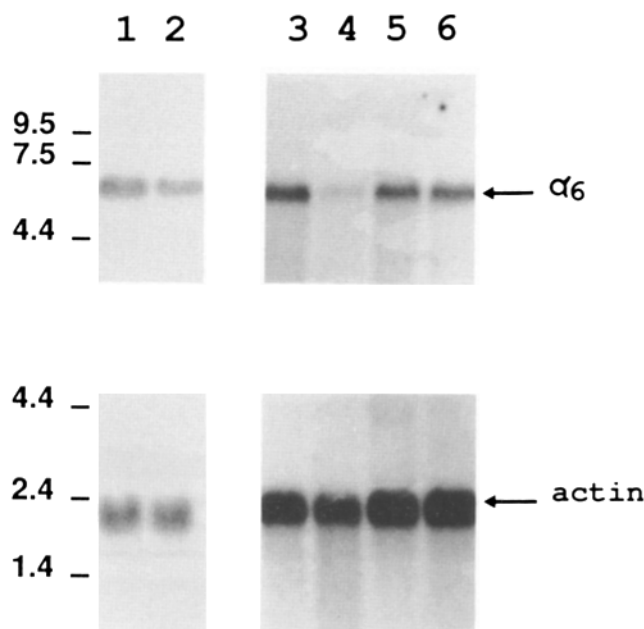


Figure 9. Distribution of α_6 mRNA in different retinal cellular fractions. Total RNA was extracted from total retinae (lanes 1 and 2), and from fractions I (lanes 3 and 4) and II (lanes 5 and 6) of E6 (lanes 1, 3, and 5) and E12 (lanes 2, 4, and 6) retinae, as described in Materials and Methods. Every sample was run in duplicate on an agarose gel, and blotted onto nitrocellulose. The blots were incubated either with a 32 P-labeled double-stranded probe obtained using the gel-purified α_6 cDNA fragment C5 (*top*), or with a 32 P-labeled actin probe (*bottom*). 20 (lanes 1 and 2) or 10 μ g (lanes 3–6) total RNA were used for each lane.

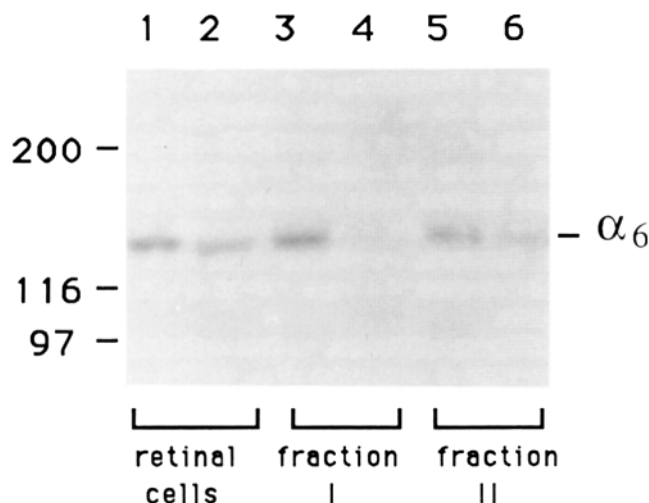


Figure 10. Distribution of α_6 protein between fractions I and II of Percoll gradients. Lysates of retinal cells and of Percoll gradient fractions from E6 (lanes 1, 3, and 5) and E12 (lanes 2, 4, and 6) chick retinae were prepared as described in Fig. 8. 100 μ g protein of each lysate was run on 6% SDS-PAGE under nonreducing conditions. After transfer to nitrocellulose, the blot was incubated with the polyclonal antibody against the cytoplasmic portion of the α_6 protein. After incubation with a 125 I-labeled anti-rabbit IgG antibody, the blot was prepared for autoradiography. Lanes 1 and 2, total retinal cells; lanes 3 and 4, fraction I (RGC fraction); lanes 5 and 6, fraction II.

mRNA and polypeptide recovered in the RGC fraction from E12 retinae may represent an overestimation due to contamination of this fraction by other retinal cells. The amount of α_6 mRNA between E12 and E6 was not dramatically different in total retina samples (Fig. 9, lanes 1 and 2) and in gradient fractions II (Fig. 9, lanes 5 and 6).

Blots of cell extracts from E6 and E12 RGC fractions from the Percoll gradients were probed with the polyclonal antibody raised against the cytoplasmic tail of α_6 . Fig. 10 shows that the amount of α_6 polypeptide present in the E12 RGC fraction was strongly reduced compared to the amount of protein found in the E6 RGC fraction (lanes 4 and 3, respectively). Quantitation from autoradiograms showed that the amount of α_6 protein in the E12 RGC fraction was only 20% of the amount found in the E6 RGC fraction. In contrast, the amount of α_6 protein detected in the total retinal cell extracts (Fig. 10, lanes 1 and 2) and in the extracts from fraction II of the gradients (Fig. 10, lanes 5 and 6) were not dramatically different between the two developmental stages. Quantitation from the autoradiogram in Fig. 10 (lanes 1 and 2) showed that the amount of α_6 protein present in E12 total retinal cells was 75% of that present in E6 total retinal cells. In contrast, cell attachment assay using retinal cells showed that <10% of E12 retinal cells adhered to LN compared to the number of adhering E6 retinal cells. Our results would thus suggest that the $\alpha_6\beta_1$ integrin must be regulated at the posttranslational level in these cells. Direct evidence for this proposal has recently been obtained in the laboratory (see Discussion).

Discussion

In previous work, the extracellular matrix protein laminin

has been shown to exert a number of dramatic influences on the survival and differentiation of neurons (reviewed in Sane, 1989; Reichardt and Tomaselli, 1991). The interactions of several neuronal subpopulations with laminin have been shown to be developmentally regulated (Cohen et al., 1986; Hall et al., 1987; Tomaselli and Reichardt, 1988). The most prominent receptors utilized by neurons to interact with laminin have been shown to be integrins (Bozyczko and Horwitz, 1986; Cohen et al., 1986; Tomaselli et al., 1986; Hall et al., 1987). These results make it interesting to characterize and study the regulation of laminin-binding integrins in neurons.

In this paper, we determine which potential laminin receptors are actually expressed by neurons in the retina, using integrin subunit-specific antibodies. Motivated by initial results demonstrating that the integrin $\alpha_6\beta_1$ is prominently expressed and has appropriate specificity to account for interactions of these neurons with laminin, we isolated and sequenced cDNA clones encoding the chick integrin α_6 subunit. Studies of α_6 mRNA and protein expression indicate that function of the $\alpha_6\beta_1$ heterodimer is regulated by two distinct mechanisms in the retina. In purified retinal ganglion cells, the one population of neurons to send axons out of the retina, expression of α_6 mRNA and protein is strongly downregulated during development and probably underlies the concurrent loss of responsiveness to laminin exhibited by these cells. In the other neurons in the embryonic retina, though, expression of α_6 mRNA and protein continues at comparatively high levels at later stages in development when these cells do not adhere to or extend neurites on laminin. These results suggest that the function of the $\alpha_6\beta_1$ integrin in the latter cells is regulated by posttranslational mechanisms, perhaps similar to mechanisms shown to regulate the adhesive function of this receptor in macrophage (Shaw et al., 1990).

Anti- β_1 antibodies have been shown to completely inhibit adhesion and neurite outgrowth of retinal neurons on LN (Hall et al., 1987). In work over the past few years, four distinct integrin α/β_1 heterodimers have been shown to mediate interactions of cells with LN. One of them, $\alpha_1\beta_1$ (Ignatius and Reichardt, 1988; Turner et al., 1989), is not a likely candidate to function as LN receptors on embryonic retinal neurons because it interacts with a region in LN not recognized by these cells. For adhesion and neurite outgrowth, retinal neurons respond to a domain near the foot of LN's cruciform structure, present in the elastase fragment E8 (Hall, D., and L. Reichardt, unpublished data). They do not interact detectably with sites in the upper portion of LN's cruciform structure present in the elastase fragments E1-4 or E1. $\alpha_1\beta_1$ has been shown to interact with sites in E1, but not with E8 (Hall et al., 1990; Tomaselli et al., 1990). We were not able to detect expression of the α_1 subunit in the retina using a specific antibody (Fig. 2).

Another potential LN receptor is the $\alpha_2\beta_1$ heterodimer, a collagen receptor that has been shown to function also as a laminin receptor in some but not all cells (Languino et al., 1989; Elices and Hemler, 1989). Using α_2 subunit-specific antibodies, we could not detect expression of this receptor in the embryonic neuroretina (Fig. 2). Consequently, it is not likely to be an important LN receptor on retinal neurons.

The $\alpha_3\beta_1$ heterodimer mediates cellular interaction with LN, FN, and, in some cells, collagen (Wayner et al., 1988;

Gehlsen et al., 1988, 1989; Tomaselli et al., 1990). The $\alpha_3\beta_1$ heterodimer binds a site near the foot of LN's cruciform structure, the same region recognized by retinal neurons (Gehlsen et al., 1989). We detected significant expression of the α_3 subunit in embryonic retina, suggesting that $\alpha_3\beta_1$ heterodimers may mediate interactions of these cells with LN. We did not detect, however, any changes in expression of the α_3 subunit that could account for the dramatic changes in LN receptor function seen during retinal development. As E12 retinal neurons are able to adhere to collagen, it is possible that $\alpha_3\beta_1$ functions as a collagen receptor in these cells. The final receptor candidate, the integrin $\alpha_6\beta_1$, is a specific LN receptor that does not bind to any other identified extracellular matrix constituent (Sonnenberg et al., 1988a). Using a subunit-specific antibody, we detected high expression of α_6 associated with β_1 in the embryonic neuroretina. In previous work, whenever the $\alpha_6\beta_1$ heterodimer has been expressed in a cell type, it has proven to be an important LN receptor (Sonnenberg et al., 1988a, 1990; Hall et al., 1990). This receptor also recognizes the same domain in LN recognized by retinal neurons (Sonnenberg et al., 1990; Hall et al., 1990; Aumailley et al., 1990). In addition, initial experiments revealed that expression of the α_6 subunit is developmentally regulated in a manner capable of explaining developmental changes in neuronal interactions with LN. We therefore focused our attention on this subunit.

With the objective of characterizing the role of the $\alpha_6\beta_1$ integrin as a neuronal LN receptor, we isolated and sequenced cDNA clones encoding this chick integrin α_6 subunit. The primary sequence of the chick α_6 subunit was shown to exhibit high homology to the primary structure of the human integrin α_6 subunit (Tamura et al., 1990). Overall, the chick and human polypeptides shared 73% identity. All features characteristic of the human α_6 subunit were conserved, including 10 potential N-linked glycosylation sites, a consensus site for cleavage of the subunit into large and small fragments, and a carboxy-terminal cytoplasmic domain which was shown to conserve 35 of 36 amino acid residues, including a consensus site for phosphorylation by Ca^{++} -calmodulin-dependent protein kinase II.

The chick integrin protein α_6 was characterized biochemically using antibodies to a peptide with the same sequence as its predicted cytoplasmic domain. When proteins were fractionated by SDS-PAGE in nonreduced conditions, an antigen of 140 kD was recognized in immunoprecipitations and immunoblots. As discussed in the results, this is the approximate size predicted by analysis of the cDNA sequence. When proteins were fractionated by SDS-PAGE in reducing conditions, a 23-kD antigen was recognized by the antibodies to the cytoplasmic domain (not shown). This corresponds to the predicted size of the small fragment of the α_6 subunit generated by proteolysis at the consensus protease cleavage site. This indicates the chick α_6 polypeptide is cleaved during maturation into large and small fragments like the human α_6 and several other α subunits (Sonnenberg, et al., 1987, 1988a; Hemler, 1990).

The α_6 subunit is able to associate with two different β subunits, β_1 or β_4 , depending primarily on whether β_4 is expressed in an individual cell type (Sonnenberg et al., 1987, 1988b, 1990; Hemler et al., 1988, 1989; Kajiji et al., 1989). While the $\alpha_6\beta_1$ integrin appears to function invariably as a LN receptor, the ligand for the $\alpha_6\beta_4$ integrin has not been

defined and recent studies have shown that several cell lines expressing this heterodimer, but not the $\alpha_6\beta_1$ integrin, do not adhere to LN (Sonnenberg et al., 1990). In immunoprecipitations using the α_6 -specific antibodies, we observed coprecipitation of α_6 with a polypeptide comigrating with the chick β_1 subunit. In contrast to immunoprecipitations using sensory neurons where virtually all of the α_6 subunit appears to be associated with β_4 (Tomaselli, K., C. Emmett, and L. Reichardt, unpublished data), we found no evidence for the presence of a large, 220-kD β_4 -like subunit in retinal neurons.

Using cDNAs and antibodies, we then studied the developmental regulation of expression of α_6 mRNA and protein levels in the retina and in subpopulations of retinal cells. These studies were motivated by previous reports showing that laminin-coated substrates promote attachment and rapid neurite outgrowth by early embryonic neuroretinal cells and retinal ganglion cells, which depends upon the function of β_1 subunit containing integrin receptors (Cohen et al., 1986; Hall et al., 1987). Between E6 and E12, both populations of neurons have been shown to lose the abilities to attach or extend neurites on laminin, but both continue to express surface integrins and remain able to interact with other extracellular matrix glycoproteins in an integrin β_1 -dependent manner (Cohen et al., 1986, 1987, 1989; Hall et al., 1987; Neugebauer et al., 1988).

As the one population of neurons to extend axons out of the retina, regulation of integrin expression in retinal ganglion cells is of special interest. For these studies, we have purified retinal ganglion cells from E6 and E12 retinas, using Percoll gradients. The purification procedure is based on the same separation principles used previously to purify this same cell population from embryonic rat retinas at different ages (Sarthy et al., 1983; Johnson et al., 1986). Microscopic examination of this fraction showed that a population of neurons with a well defined morphology was recovered in this fraction (fraction I). Biochemical analysis showed that only this fraction was enriched in the chick L1/NgCAM/8D9/G4 antigen, a cell adhesion molecule shown previously to be a marker for retinal ganglion cells in both mammalian and avian embryos (Lemmon and McLoon, 1986; Pigott and Davies, 1987). Reproducing previous results, we showed that E6 retinal ganglion cells, but not E12 retinal ganglion cells extend long processes on laminin-coated substrates and process growth is completely abolished by the anti-integrin β_1 subunit-specific monoclonal antibody CSAT. When protein levels were measured, only 20% as much α_6 antigen was detected in E12 as in E6 retinal ganglion cells (Fig. 10). Our results showed also that the reduction of α_6 expression reflects reduced levels of α_6 mRNA. Only 25% as much α_6 mRNA was found in E12 compared to E6 retinal ganglion cells (Fig. 9). In fact, these measurements may overestimate the amount of α_6 mRNA and protein present in E12 retinal ganglion cells, since any cells contaminating the retinal ganglion cell-enriched fraction express high levels of both α_6 mRNA and protein (see below).

The results suggest that developmental changes in the ability of retinal ganglion cells to interact with LN reflect, in large part, changes in gene expression. They are consistent with binding experiments using ^{125}I -LN which have demonstrated a fivefold decrease between E6 and E12 in the number of LN-binding sites present on the surfaces of purified chick

retinal ganglion cells (Cohen et al., 1989). It is possible that additional, post-translational mechanisms regulate the activity of the remaining integrin $\alpha_6\beta_1$ heterodimers present on these cells (Shaw et al., 1990), as it has been recently shown for $\alpha_5\beta_1$ in keratinocytes (Adams and Watt, 1990).

During embryogenesis, the first axons of retinal ganglion cells enter the optic nerve at about E3 (Thanos and Bonhoeffer, 1983). Most retinal ganglion cell axons enter the optic nerve between E6 and E10 (Rager, 1980). By E12, the axons of virtually all retinal ganglion cells have reached the optic tectum. The time course with which retinal ganglion cell axons invade the optic nerve and tectum correlate reasonably well with the time course of loss of responsiveness of these neurons to LN (Cohen et al., 1989). During this same period, isoforms of LN can be detected in the interior of the optic nerve, which disappear at later developmental stages (Cohen et al., 1987). The transient expression of LN in the retinal-tectal pathway thus also correlates well with the period during which retinal ganglion cell axon outgrowth occurs. These results suggest that regulation of expression of LN in the optic nerve and of integrin α subunit gene expression in retinal ganglion cells both help determine the behavior of growth cones in the retinal-tectal pathway.

Mechanisms by which expression of genes encoding integrin subunits are regulated in neurons are poorly understood. In the retinal-tectal system, ablation of the tectum at E6 has been shown to result in persistent expression of active LN receptors in retinal ganglion cells at E11, suggesting that tectal signals inhibit LN receptor function in these cells (Cohen et al., 1989). It will be interesting to determine whether tectal ablation enhances α_6 gene expression in retinal ganglion cells and if BDNF, a trophic factor for these neurons (Johnson et al., 1986), regulates α_6 gene expression.

The vast majority of retinal cells are not retinal ganglion cells and do not extend axons out of the retina. Nonetheless, virtually all of these neurons interact with LN at early (E6), but not late (E12) developmental stages (Hall et al., 1987). These neurons continue to express the integrin β_1 subunit and remain able to attach to collagen, using β_1 subunit-containing integrins (Hall et al., 1987). In the present paper, we examined expression of α_6 mRNA and protein in both total retinal cells, and in retinal cells separated from retinal ganglion cells by Percoll gradient sedimentation (fraction II). In both total retinal cells and in cells from fraction II, a comparatively small decline in expression of α_6 mRNA and protein was seen between E6 and E12 (Figs. 9 and 10). While the decreases appear to be significant, they do not appear large enough to account for the failure of the older cell populations to interact with LN. It seems likely, therefore, that the function of $\alpha_6\beta_1$ receptors on these cells is regulated by a posttranslational mechanism, possibly similar to that regulating integrin function in macrophage or keratinocytes (Shaw et al., 1990; Adams and Watt, 1990). This data is consistent with binding experiments using ^{125}I -LN which detected a change in affinity, but not in number of binding sites on these cells between E6 and E12 (Cohen et al., 1989). Providing direct support for this model, an integrin β_1 subunit-specific mAb named TASC, which enhances ligand binding by many β_1 integrins, has also been shown to restore the ability of 80% of E12 retinal cells to attach to LN (Neugebauer, K. M., and L. F. Reichardt, manuscript submitted for publication).

In summary, results presented in this paper suggest that integrin function is regulated at two different levels in the embryonic retina. In the future, it will be interesting to identify the molecules and mechanisms that cause these changes. It will also be important to understand how these regulatory events direct differentiation of cells in the retina and movements of growth cones in the optic nerve and tectum.

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Note Added in Proof. In more recent experiments, an antibody has been prepared to a chick integrin α_6 fusion protein purified from an *E. coli* lysate. These antibodies inhibit dramatically neurite outgrowth by retinal neurons on laminin, providing more direct evidence for the functional role of the $\alpha_6\beta_1$ integrin in the retina.

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